

FINAL REPORT

Test Facility Study No. 511881

**Acute Toxicity Study in *Daphnia Magna* with
MLA-3202
(Static)**

SPONSOR:

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03 April 2017

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1. STATEMENT OF GLP COMPLIANCE

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

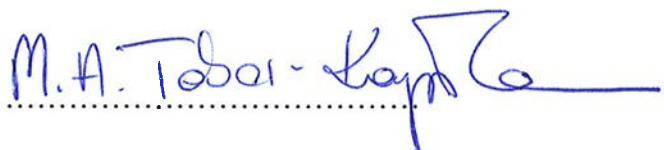
All phases of this study performed by the test facility were conducted in compliance with:

- OECD Principles of Good Laboratory Practice;
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

The data generated and reported are considered to be valid.

Charles River Den Bosch

Signature:



Name: M.A. Tobor-Kaplon, PhD.

Title: Study Director

Date: 03 April 2017

2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands.

Study title: Acute toxicity study in *Daphnia magna* with MLA-3202 (static)

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Project	511881			
Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study	Study Plan	10-Aug-2016	10-Aug-2016	10-Aug-2016
	Study Plan Amendment 01	27-Oct-2016	27-Oct-2016	27-Oct-2016
	Study Plan Amendment 02	06-Jan-2017	06-Jan-2017	06-Jan-2017
	Report	13-Mar-2017	13-Mar-2017	13-Mar-2017
Process	Environmental Science	03-Oct-2016	18-Oct-2016	18-Oct-2016
	Test Substance Handling			
	Exposure			
	Observations/Measurements			
	Test Substance Receipt	21-Nov-2016	21-Nov-2016	21-Nov-2016
	Test Substance Handling			
	Analytical and physical chemistry	01-Dec-2016	28-Dec-2016	28-Dec-2016
	Test Substance Handling			
	Exposure			
	Observations/Measurements			
	Specimen Handling			
	Environmental Science	09-Jan-2017	23-Jan-2017	23-Jan-2017
	Test Substance Handling			
	Exposure			
	Observations/Measurements			

*TFM=Test Facility Management SD = Study Director

The facility inspection program is conducted in accordance with Standard Operating Procedure.

The review of the final report was completed on the date of signing this QA statement.

Charles River Den Bosch

Signature:



Name: **Bart Kluskens, BSc**
Quality Assurance Auditor

Date:



3. SUMMARY

Acute Toxicity Study in *Daphnia magna* with MLA-3202 (Static).

The study procedures described in this report were based on the OECD guideline No. 202, 2004. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.2, 2008.

The batch of MLA-3202 tested was a clear amber-red liquid completely soluble in test medium at the concentrations tested. The test item was a UVCB substance.

A full test was performed based on the results of a combined limit/range-finding test and a range-finding test. Twenty daphnids per group (5 per replicate, quadruplicate) were exposed to an untreated control and to 0.10, 0.18, 0.32, 0.56 and 1.0 mg/L. The total exposure period was 48 hours and samples for analytical confirmation of exposure concentrations were taken at the start and at the end of the test.

Samples taken from all test concentrations were analyzed. Measured concentrations were at the level of nominal at the start of the test (85-91%) and decreased to 10-84% of initial at the end of the exposure period. Based on these results, the average exposure concentrations were 0.029, 0.10, 0.22, 0.44 and 0.81 mg/L in nominally 0.10, 0.18, 0.32, 0.56 and 1.0 mg/L, respectively.

The study met the acceptability criteria prescribed by the study plan and was considered valid.

The 48h-EC₅₀ was 0.14 mg/L based on average exposure concentrations (95% confidence interval between 0.12 and 0.17 mg/L).

4. INTRODUCTION

4.1. Study schedule

Experimental starting date : 21 November 2016
Experimental completion date : 26 January 2017

4.2. Purpose

The purpose of the study was to evaluate the test item for its ability to generate acute toxic effects on the mobility of *Daphnia magna* during an exposure period of 48 hours and, if possible, to determine the EC₅₀ at 24 and 48 hours of exposure.

4.3. Guidelines

The study procedures described in this report are in compliance with the Organization for Economic Co-operation and Development (OECD), OECD guidelines for Testing of Chemicals, guideline No. 202: "*Daphnia* sp., Acute Immobilisation Test", Adopted April 13, 2004.

In addition, the procedures were designed to meet the test methods of the following guideline:

- Council Regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.2. "*Daphnia* sp. Acute Immobilisation Test".

4.4. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

4.5. Responsible personnel

4.5.1. Test facility

Study Director M.A. Tobor-Kaplon, PhD.
Principal Scientist (Analytical Chemistry) M.J.C. Brekelmans, MSc.

4.5.2. Sponsor Representative

Study Monitor Audrey Batoon, PhD.

4.6. Definitions

Immobile are those animals not able to swim within 15 seconds after gentle agitation of the test vessel.

The **EC₅₀** is the concentration of test item estimated to immobilise 50% of the daphnids after a defined period of exposure.

5. MATERIALS AND METHODS

5.1. Test item

5.1.1. Test item information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)

See [APPENDIX 2](#): Certificate of analysis.

5.1.2. Study specific test item information

Purity/composition correction factor	No correction factor required
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

5.2. Vehicle information

Solubility in water:	< 1 g/L
Stability in water:	Yes

5.3. Reference item

This report includes the results of the most recent reference test with potassium dichromate ([APPENDIX 3](#)).

5.4. Preparation of test solutions

The batch of MLA-3202 tested was a clear amber-red liquid completely soluble in test medium at the concentrations tested. The test item was a UVCB substance. No correction was made for the purity/composition of the test item.

Preparation of test solutions started with the highest concentration of 1 mg/L applying one hour of magnetic stirring to accelerate the dissolution of the test item in the test medium. The lower test concentrations were prepared by subsequent dilutions of the highest concentration in test medium. All final test solutions were clear and colourless.

Note that in the combined limit/range-finding test the highest test concentration was 100 mg/L and was stirred for a period of 2.5 hours. The highest concentration in this test was hazy while the remaining concentrations were clear and colorless. In the range-finding test, the highest test concentration was 10 mg/L. All solutions were clear and colorless.

5.5. Test system

Species	<i>Daphnia magna</i> (Crustacea, Cladocera) (Straus, 1820), at least third generation, obtained by a cyclical parthenogenesis under specified breeding conditions.
Source	In-house laboratory culture with a known history.
Reason for selection	This system has been selected as an internationally accepted invertebrate species.

Validity of batch	Daphnids originated from a healthy stock, 2 nd to 5 th brood, showing no signs of stress such as mortality >20%, presence of males, ephippia or discoloured animals and there was no delay in the production of the first brood.
Characteristics	For the test, young daphnids with an age of < 24 hours were selected from parental daphnids older than two weeks.

5.6. Breeding

Start of each batch	With newborn daphnids, i.e. less than 3 days old, by placing about 250 of them into 5 litres of medium in an all-glass culture vessel.
Maximum age of the cultures	4 weeks
Renewal of the cultures	After 7 days of cultivation half of the medium twice a week.
Temperature of medium	18-22°C
Feeding	Daily, a suspension of fresh water algae.
Medium	M7, as prescribed by Dr. Elendt-Schneider (Elendt, B.-P., 1990: Selenium deficiency in Crustacea. An ultrastructural approach to antennal damage in <i>Daphnia magna</i> Straus. <i>Protoplasma</i> 154, 25-33).

Composition of medium M7:

Adjusted ISO medium: the following chemicals (analytical grade) are dissolved in tap water purified by Reverse Osmosis (RO-water, GEON Waterbehandeling, Berkel-Enschot, The Netherlands):

Macro salts:	CaCl ₂ .2H ₂ O	211.5	mg/L
	MgSO ₄ .7H ₂ O	88.8	mg/L
	NaHCO ₃	46.7	mg/L
	KCl	4.2	mg/L
Medium M7: trace elements, macronutrients and vitamins are added to freshly prepared ISO medium to reach the following concentrations:			
Trace elements:	B	0.125	mg/L
	Fe	0.05	mg/L
	Mn	0.025	mg/L
	Li, Rb and Sr	0.0125	mg/L
	Mo	0.0063	mg/L
	Br	0.0025	mg/L
	Cu	0.0016	mg/L
	Zn	0.0063	mg/L
	Co and I	0.0025	mg/L
	Se	0.0010	mg/L
	V	0.0003	mg/L
	Na ₂ EDTA.2H ₂ O	2.5	mg/L
Macro nutrients:	Na ₂ SiO ₃ .9H ₂ O	10.0	mg/L
	NaNO ₃	0.27	mg/L
	KH ₂ PO ₄	0.14	mg/L
	K ₂ HPO ₄	0.18	mg/L
Vitamins:	Thiamine	75.0	µg/L

B ₁₂	1.0	µg/L
Biotin	0.75	µg/L

The hardness: 180 mg/L expressed as CaCO₃ and the pH: 7.7 ± 0.3.

5.7. Combined limit/range-finding test

The project started with a combined limit/range-finding test. Twenty daphnids per concentration (four replicates, 5 daphnids per vessel) were exposed to a control and 100 mg/L. Test procedure and conditions were similar to those applied in the full test with the following exceptions:

- Ten daphnids per concentration (in duplicate, 5 per vessel) were exposed to 0.10, 1.0 and 10 mg/L in the combined range-finding test.
- Dissolved oxygen concentrations and pH were only measured in the control and the highest test concentration.

5.8. Range-finding test

A range-finding test was performed to provide confirmation about the range of concentrations to be used in the final test. Test procedure and conditions were similar to those applied in the full test with the following exceptions:

- Ten daphnids per concentration (in duplicate, 5 per vessel) were exposed to a range of 0.10 to 10 mg/L increasing by a factor of 10 and to a control.
- Dissolved oxygen concentrations and pH were only measured in the control and the highest test concentration.
- No sampling for determination of actual test concentrations was performed.

5.9. Final test

5.9.1. Test concentrations

MLA-3202	0.10, 0.18, 0.32, 0.56 and 1.0 mg/L
Control	Test medium without test item or other additives.

5.9.2. Test procedure and conditions

Test duration	48 hours
Test type	Static
Test vessels	100 mL, all-glass
Medium	Adjusted ISO medium
Number of daphnids	20 per concentration
Loading	5 per vessel containing 80 mL of test solution
Light	16 hours photoperiod daily
Feeding	No feeding
Aeration	No aeration of the test solutions.
Introduction of daphnids	Within 36 minutes after preparation of the test solutions.

5.9.3. Sampling for analysis of test concentrations

Single samples for possible analysis were taken from all test concentrations and the control according to the schedule below. The method of analysis is described in the appended Analytical Report ([APPENDIX 4](#)).

Frequency	at t=0 h and t=48 h
Volume	1.0 mL from the approximate centre of the test vessels
Storage	Samples were stored in a freezer ($\leq -15^{\circ}\text{C}$) until analysis.

At the end of the exposure period, the replicates were pooled at each concentration before sampling.

Additionally, single reserve samples of 1.0 mL were taken for possible analysis. If not used, these samples were stored in a freezer ($\leq -15^{\circ}\text{C}$) for possible analysis until delivery of the final report with a maximum of three months.

5.9.4. Measurements and recordings

Immobility (including mortality)	At 24 hours and at 48 hours.
pH and dissolved oxygen	At the beginning and at the end of the test, for all concentrations and the control.
Temperature of medium	Continuously in a temperature control vessel, beginning at the start of the test.

5.10. Interpretation

5.10.1. Data handling

Determination of the average exposure concentrations:

The average exposure concentrations were calculated as $\sqrt{C_{t=0} \times C_{t=48}}$, being the geometric means of the concentrations of MLA-3202 measured in the samples taken at the start ($C_{t=0}$) and the end of the test ($C_{t=48}$).

Calculation of EC₅₀:

The 24 and 48h-EC₅₀-value was calculated from the probits of the percentages of affected daphnids and the logarithms of the corresponding test item average exposure concentrations using the maximum likelihood estimation method.

ToxRat Professional v 3.2.1 (ToxRat Solutions® GmbH, Germany) was used to perform the analyses.

5.10.2. Acceptability of the test

1. In the control, no daphnids became immobilised or showed other signs of disease or stress, for example discolouration or unusual behaviour such as trapping at the surface of the medium.
2. The oxygen concentration at the end of the test was ≥ 3 mg/L in control and test vessels.

5.11. List of deviations

5.11.1. List of study plan deviations

1. During the range-finding test, 10 daphnids were exposed to a control instead of 20 indicated by the protocol.
Evaluation: By mistake this was not correctly indicated in the study plan while use of 10 daphnids in a range-finding test is a standard procedure.
2. In the full test, oxygen concentration was not measured for the test concentration at which effects were complete after 24 hours of exposure.
Evaluation: Oxygen concentration was measured at the end of the test and was > 3 mg/L.

The study integrity was not adversely affected by the deviations.

5.11.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA): Temperature.

7. RESULTS

7.1. Combined limit/range-finding test

Table 1 shows the responses recorded during the combined limit/range-finding test. At the end of the exposure period, more than 10% of the daphnids were observed to be immobile in the control group, therefore the test was considered not valid. Nevertheless, the responses were used to set the range of concentrations to be tested in the range-finding test. No immobility was observed at the lowest test concentration throughout the test duration, while effects were complete already after 24 hours of exposure at the three highest test concentrations.

Samples taken from nominally 0.10 and 1.0 mg/L were analysed. The actual concentrations were 0.10 and 1.1 mg/L at the start of the test, respectively. These concentrations decreased to 23-74% of initial at the end of the test (see also **Table 2** of the appended Analytical Report).

All test conditions were maintained within the limits prescribed by the study plan.

Table 1
Number of introduced daphnids and incidence of immobility in the combined limit/range-finding test

Time (h)	Replicate	Nominal concentration MLA-3202 (mg/L)				
		Control	0.1	1.0	10	100
0	A	5	5	5	5	5
	B	5	5	5	5	5
	C	5			5	
	D	5			5	
	Total introduced	20	10	10	10	20
24	A	0	0	5	5	5
	B	0	0	5	5	5
	C	0			5	
	D	0			5	
	Total immobilised	0	0	10	10	20
48	Effect %	0	0	100	100	100
	A	1	0	5	5	5
	B	1	0	5	5	5
	C	0			5	
	D	1			5	
Total immobilised		3	0	10	10	20
	Effect %	15	0	100	100	100

7.2. Range-finding test

The highest concentration tested in the range-finding test was 10 mg/L. This concentration was clear and colorless at the end of the preparation period unlike the concentration of 100 mg/L prepared in the preceding test.

Table 2 shows the responses recorded during the range-finding test. No immobility was observed in the control and at the lowest test concentration during the test period, while effects were complete already after 24 hours at the two higher test concentrations. Therefore, the 48h-EC₅₀ was expected between 0.10 and 1.0 mg/L.

Microscopic observations of daphnids exposed to 1.0 mg/L (after 24h and 48h of exposure) and 10 mg/L (after 48h of exposure) revealed no test item attached to their bodies.

All test conditions were maintained within the limits prescribed by the study plan.

Table 2
Number of introduced daphnids and incidence of immobility in the range-finding test

Time (h)	Replicate	Nominal concentration MLA-3202 (mg/L)			
		Control	0.10	1.0	10
0	A	5	5	5	5
	B	5	5	5	5
	Total introduced	10	10	10	10
24	A	0	0	5	5
	B	0	0	5	5
	Total immobilised	0	0	10	10
	Effect %	0	0	100	100
48	A	0	0	5	5
	B	0	0	5	5
	Total immobilised	0	0	10	10
	Effect %	0	0	100	100

7.3. Final test

7.3.1. Measured concentrations

The results of analysis of the samples taken during the final test are described in [Table 3](#) of the appended Analytical Report.

Samples taken from all test concentrations were analysed. Measured concentrations were at the level of nominal at the start of the test (85-91%) and decreased to 10-84% of initial at the end of the exposure period. Therefore, the average exposure concentrations were calculated and used to determinate the effect parameters. (see [Table 3](#)).

Table 3
Average exposure concentration versus nominal concentration

Nominal concentration (mg/L)	Measured concentrations (mg/L)		Average exposure (mg/L)
	t=0h	t=48h	
0.10	0.0909	0.00937	0.029
0.18	0.154	0.0672	0.10
0.32	0.284	0.171	0.22
0.56	0.493	0.398	0.44
1.0	0.882	0.742	0.81

7.3.2. Immobility

[Table 4](#) shows the responses recorded during the final test. No immobility was observed in the control and at the lowest test concentration, while effects were complete already after 24 hours of exposure at the highest test concentration. After 24 hours of exposure, no daphnids were immobile at 0.10 mg/L while 35 and 90% were immobile at 0.22 and 0.44 mg/L, respectively. At the end of the exposure period, 20% and 85% of daphnids were immobile at 0.10 and 0.22 mg/L, respectively, while at the two highest concentrations the effects were complete.

The responses recorded in this test allowed for reliable determination of an EC₅₀.

Table 4
Number of introduced daphnids and incidence of immobility in the final test

Time (h)	Replicate	MLA-3202; Average exposure concentrations (mg/L)					
		Control	0.029	0.10	0.22	0.44	0.81
0	A	5	5	5	5	5	5
	B	5	5	5	5	5	5
	C	5	5	5	5	5	5
	D	5	5	5	5	5	5
	Total introduced	20	20	20	20	20	20
24	A	0	0	0	1	4	5
	B	0	0	0	1	5	5
	C	0	0	0	2	5	5
	D	0	0	0	3	4	5
	Total immobilised	0	0	0	7	18	20
48	Effect %	0	0	0	35	90	100
	A	0	0	0	4	5	5
	B	0	0	1	4	5	5
	C	0	0	1	4	5	5
	D	0	0	2	5	5	5
Total immobilised	0	0	4	17	20	20	20
	Effect %	0	0	20	85	100	100

7.3.3. Determination of effect concentrations

Table 5 shows the effect parameters based on average exposure concentrations, see also APPENDIX 1.

Table 5
Effect parameters

Parameter	MLA-3202	
	Average exposure concentration (mg/L)	95%-confidence interval (mg/L)
24h-EC ₅₀	0.26	0.22-0.31
48h-EC ₅₀	0.14	0.12-0.17

7.3.4. Experimental conditions

The measured pH and oxygen concentrations (mg/L) are presented in Table 6. These test conditions remained within the limits prescribed by the study plan (pH: 6.0-9.0, not varying by more than 1.5 units; oxygen: ≥ 3 mg/L at the end of the test).

The temperature continuously measured in a temperature control vessel remained stable at 20°C during the test, and complied with the requirements as laid down in the study plan (18-22°C, constant within 2°C).

Table 6
pH and oxygen concentrations (mg/L) during the final test

Average exposure concentration (mg/L)	MLA-3202		Start (t=0 h)		End (t=48 h)	
		pH	O ₂	pH	O ₂	
Control		7.8	9.7	8.0	9.3	
0.029		7.8	9.6	8.0	9.2	
0.10		7.8	9.5	7.9	9.3	
0.22		7.8	9.5	7.9	9.2	
0.44		7.8	9.4	7.9	9.2	
0.81		7.8	9.3	7.9	9.2	

8. CONCLUSION

The 48h-EC₅₀ was 0.14 mg/L based on average exposure concentrations (95% confidence interval between 0.12 and 0.17 mg/L).

APPENDIX 1**EC-VALUES****Table 7****Parameters of the probit analysis at 24h**

Parameter	Value
Computation runs:	8.00000
Slope b:	6.04174
Intercept a:	3.52076
Variance of b:	1.60104
Goodness of Fit	
Chi ² :	0.25280
Degrees of freedom:	3.00000
p(Chi ²):	0.96900
Log LC ₅₀ :	-0.58274
SE Log LC ₅₀ :	0.03776
g-Criterion:	0.16849
F:	270.55700
p(F) (df: 1;3):	<0.001

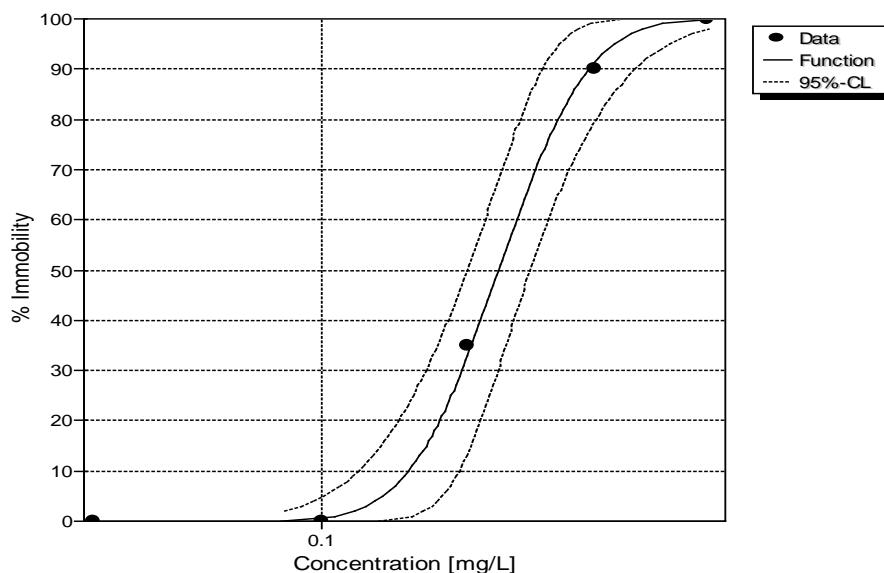


Figure 1
Percentage response (=immobility) of Daphnia magna as function of concentration of
MLA-3202 at 24h

Table 8
Results of the probit analysis at 24h

Parameter	EC₅₀
Value [mg/L]	0.261
lower 95%-cl	0.220
upper 95%-cl	0.310

APPENDIX 1
EC-VALUES – continued –
Table 9

Parameters of the probit analysis at 48h

Parameter	Value
Computation runs:	8.00000
Slope b:	5.64660
Intercept a:	4.78844
Variance of b:	1.52997
Goodness of Fit	
Chi ² :	0.07219
Degrees of freedom:	3.00000
p(Chi ²):	0.99500
Log LC ₅₀ :	-0.84802
SE Log LC ₅₀ :	0.04138
g-Criterion:	0.18433
F:	866.02200
p(F) (df: 1;3):	<0.001

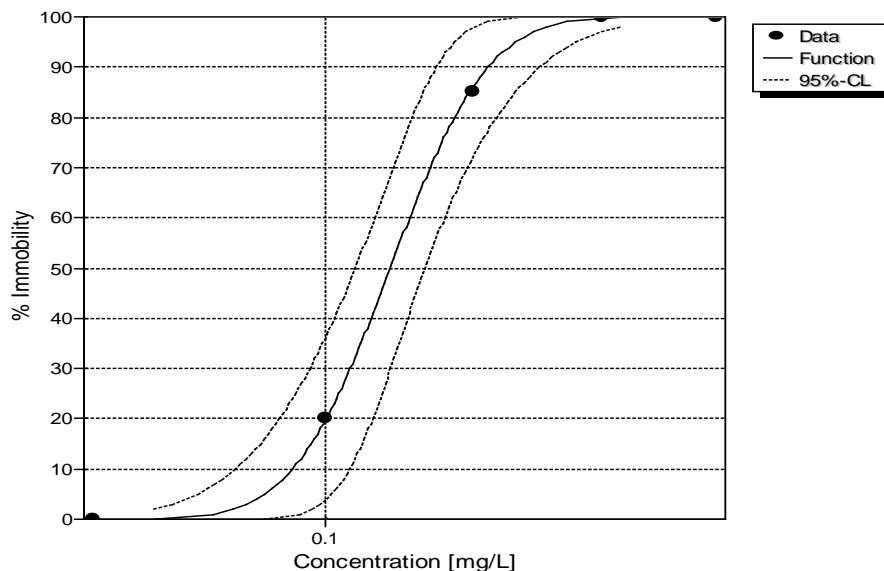


Figure 2
Percentage response (=immobility) of Daphnia magna as function of the concentration of MLA-3202 at 48h

Table 10
Results of the probit analysis at 48h

Parameter	EC ₅₀
Value [mg/L]	0.142
lower 95%-cl	0.118
upper 95%-cl	0.171

APPENDIX 2
CERTIFICATE OF ANALYSIS



Chemtura Corporation
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C ₂₄ H ₄₇ NO ₃	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C ₂₂ H ₄₅ NO ₃	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C ₂₄ H ₄₅ NO ₃	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C ₂₄ H ₄₉ NO ₃	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C ₂₂ H ₄₃ NO ₃	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C ₂₆ H ₄₅ NO ₃	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C ₂₄ H ₄₃ NO ₃	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C ₁₈ H ₃₄ O ₂	C18:1 (oleic) acid
1.1	421.4	C ₂₆ H ₄₇ NO ₃	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis 3/7/16
 Blake Lewis Date
 Analytical REACH Scientist, Analytical Services
Colin Moore 3/7/16
 Albert J. Nitowski Date
 Sr. Technology Manager
 Analytical and Lab Support Services

APPENDIX 3
REFERENCE TEST

Start: 14 November 2016

End: 16 November 2016

48-hour Acute Toxicity Study in *Daphnia magna* with Potassium Dichromate ($K_2Cr_2O_7$) (Project 515957).

The study procedures described in this report were based on the OECD guideline No. 202: "Daphnia sp., Acute Immobilisation Test", Adopted April 13, 2004 and the ISO International Standard 6341.

The reference test was carried out to check the sensitivity of the test system as used by Charles River Den Bosch. Daphnids were exposed for a maximum of 48 hours to $K_2Cr_2O_7$ concentrations of 0.10, 0.18, 0.32, 0.56, 1.0 and 1.8 mg/L and to a control. Twenty daphnids were exposed per concentration.

The reference item, potassium dichromate ($K_2Cr_2O_7$, art. 1.04864, batch no. K44879664) was obtained from Merck, Darmstadt, Germany.

Acute immobilization of daphnia after 24 and 48 hours in the reference test with potassium dichromate:

Potassium Dichromate Nominal conc. (mg/L)	Number Exposed	% immobile		Expected response (%) After 48 hours ¹	
		24h	48h	Minimal	Maximal
control	20	0	0	0	10^2
0.10	20	0	0	0	10
0.18	20	0	0	0	10
0.32	20	5	15	0	30
0.56	20	5	40	0	100
1.0	20	65	100	40	100
1.8	20	100	100	100	100

¹ Based on historical data of the previous years (n>60).

² A maximum response of 10% does not invalidate the results of the test.

The actual responses in this reference test with $K_2Cr_2O_7$ were within the ranges of the expected responses at the different concentrations, i.e. the 48h-EC₅₀ was within the expected range of 0.28 to 0.9 mg/L. Hence, the sensitivity of the daphnia was within the range determined with the historical data collected at Charles River Den Bosch.

The 24h-EC₅₀ was 0.91 mg/L with a 95% confidence interval ranging from 0.77 to 1.1 mg/L.

The 48h-EC₅₀ was 0.58 mg/L with a 95% confidence interval ranging from 0.49 to 0.67 mg/L.

The study plan, raw data and report from this study are kept in the Charles River Den Bosch archives. The test described above was performed under GLP with a QA-check.

APPENDIX 4
ANALYTICAL REPORT

**Determination of the
concentrations**

Author

M.J.C. Brekelmans, MSc.

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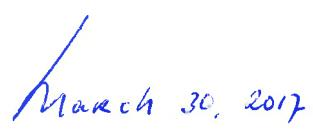
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REPORT APPROVAL



M.J.C. Brekelmans, MSc.
Individual Scientist

Date:



1. INTRODUCTION

The objective of this analytical study was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

For the work detailed in this report, the experimental start date was 05 Dec 2016, and the experimental completion date was 26 Jan 2017.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Text Table 1
Chemicals and Reagents

Chemical / Reagent	Supplier
Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Methanol	Biosolve, Valkenswaard, The Netherlands
Ammonium acetate	Biosolve
ISO-medium	See main report

All chemicals and reagents were of analytical grade, unless specified otherwise.

2.2. Methods

2.2.1. Analytical Method

Analysis was based on the analytical method validated for the test item in project 511870.

Analytical conditions

Instrument	Acquity UPLC system (Waters, Milford, MA, USA)
Detector	Xevo TQ-S mass spectrometer (Waters)
Column	Acquity UPLC HSS Cyano, 100 mm × 2.1 mm i.d., dp = 1.8 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	5 µL
Mobile phase	10 mM Ammonium acetate in 70/30 (v/v) methanol/water
Flow	0.4 mL/min
MS detection	
Ionisation source	ESI ⁺
Cone voltage	50 V
Acquisition	m/z 398.2 → m/z 134 (Collision energy 18 eV) m/z 372.2 → m/z 134 (Collision energy 16 eV) m/z 396.2 → m/z 134 (Collision energy 16 eV) m/z 400.3 → m/z 134 (Collision energy 18 eV)
Quantitation	m/z 398.2 → m/z 134

2.2.2. Test Samples

Test samples were stored in the freezer ($\leq -15^{\circ}\text{C}$). Storage stability of samples under these conditions was demonstrated in project 511870.

On the day of analysis, the test samples were thawed at room temperature. The samples were diluted in a 1:3 (v:v) ratio with methanol and analyzed. If necessary, the samples were further diluted with 75/25 (v/v) methanol/ISO-medium to obtain concentrations within the calibration range.

2.2.3. Preparation of Solutions

Stock and Spiking Solutions

Stock solutions of the test item were prepared in methanol at a concentration of 2000 mg/L.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was methanol.

Calibration Solutions

Solutions with the test item in the concentration range of 200 - 30000 $\mu\text{g}/\text{L}$ were prepared in methanol from two stock solutions. The solutions were 100-fold diluted with 75/25 (v/v) methanol/ISO-medium to obtain calibration solutions in the concentration range of 2 - 300 $\mu\text{g}/\text{L}$.

Quality Control (QC) Samples

1 mL blank medium was spiked with the test item at a target concentration of 0.01 or 10 mg/L. The QC samples were treated similarly as the test samples (see paragraph [2.2.2](#) ‘Test Samples’).

Blank QC samples consisting of blank medium were treated similarly to the QC and test samples.

2.2.4. Sample Injections

Calibration solutions were injected in duplicate. Test samples and QC samples were analyzed by single injection.

3. CONSTRUCTED VARIABLES

Response (R)

Peak area test item [units]

Calibration curve

$$R = a C_N + b$$

where:

 C_N = nominal concentration [$\mu\text{g}/\text{L}$]a = slope [units $\times \text{L}/\mu\text{g}$]

b = intercept [units]

Analyzed concentration (C_A)

$$C_A = \frac{(R - b)}{a} \times \frac{d}{1000} \quad [\text{mg}/\text{L}]$$

where:

d = dilution factor

Accuracy

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to nominal concentration

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to initial

$$\frac{C_A (t = x \text{ hours})}{C_A (t = 0 \text{ hours})} \times 100 \quad [\%]$$

Limit of detection (LOD)

$$LOD = \frac{3N}{S} \times C_N$$

where:

 N = noise height [units] S = peak height [units]

4. COMPUTERIZED SYSTEMS

Critical computerized systems used in the phase are listed below. All computerized systems used in the conduct of this phase have been validated.

Text Table 2
Critical Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
MassLynx	4.1	System control, data acquisition and processing
REES Centron	SQL 2.0	Temperature, relative humidity and/or atmospheric pressure monitoring

5. RESULTS

5.1. Calibration Curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration² weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

5.2. Samples

5.2.1. QC Samples

The results for the QC samples are given in [Table 1](#).

A small response at the retention time of the test item was detected in the chromatograms of the blank QC samples. Concentration was below the limit of detection which was 0.00029 mg/L during the range-finding test and 0.00012 mg/L during the final test.

During the range-finding test, the mean accuracy of the 0.01 mg/L QC samples was slightly above the criterion range of 70-110% (i.e. 111% of target). The mean accuracy of the 10 mg/L QC samples was within the criterion range. During the final test, the mean accuracies of QC samples containing test item fell within the criterion of 70-110%. It demonstrated that the analytical method was adequate for the determination of the test item concentration in the test samples.

5.2.2. Test Samples

The results for the test samples are given in [Table 2](#) and [Table 3](#).

Table 1
QC Samples

Date of preparation	Date of analysis	Concentration [mg/L]			Accuracy [%]	
		Target	Nominal	Analyzed	Individual	Mean
05 Dec 2016	05 Dec 2016	0	0.00	< LOD	n.a.	n.a.
			0.00	< LOD	n.a.	
05 Dec 2016	05 Dec 2016	0.01	0.0100	0.0114	114	111
			0.0100	0.0107	107	
05 Dec 2016	05 Dec 2016	10	10.0	9.42	94	99
			10.0	10.3	103	
26 Jan 2017	26 Jan 2017	0	0.00	< LOD	n.a.	n.a.
			0.00	< LOD	n.a.	
26 Jan 2017	26 Jan 2017	0.01	0.0100	0.0102	102	102
			0.0100	0.0102	102	
26 Jan 2017	26 Jan 2017	10	10.0	9.87	99	100
			10.0	10.0	100	

LOD The limit of detection of the method, taking a dilution factor of four into account, was determined to be 0.00029 mg/L on 05 Dec 2016 and 0.00012 mg/L on 26 Jan 2017.

n.a. Not applicable.

Table 2
Range-finding Test: Test Samples

Time of sampling [hours]	Date of sampling	Date of analysis ¹	Concentration [mg/L]		Relative to nominal [%]	Relative to initial [%]
			Nominal	Analyzed		
0	21 Nov 2016	05 Dec 2016	0.1	0.102	102	
			1.0	1.12	112	
48	23 Nov 2016	05 Dec 2016	0.1	0.0240	24	23
			1.0	0.826	83	74

¹ Samples were stored in the freezer ($\leq -15^{\circ}\text{C}$) until the day of analysis.

Table 3
Final Test: Test Samples

Time of sampling [hours]	Date of sampling	Date of analysis ¹	Concentration [mg/L]		Relative to nominal [%]	Relative to initial [%]
			Nominal	Analyzed		
0	16 Jan 2017	26 Jan 2017	0	< LOD		
			0.10	0.0909	91	
			0.18	0.154	85	
			0.32	0.284	89	
			0.56	0.493	88	
			1.0	0.882	88	
48	18 Jan 2017	26 Jan 2017	0	< LOD		
			0.10	0.00937	9.4	10
			0.18	0.0672	37	44
			0.32	0.171	53	60
			0.56	0.398	71	81
			1.0	0.742	74	84

¹ Samples were stored in the freezer ($\leq -15^{\circ}\text{C}$) until the day of analysis.

LOD The limit of detection of the method, taking a dilution factor of four into account, was determined to be 0.00012 mg/L.